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SIMPLE EXPLANATION OF THE MISSES IN THE COOPERATION OF CHARGES IN PHOTOSYNTHETIC O_2 EVOLUTION

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Abstract—In the model of Forbush *et al.* (1971) the observed damping of the flash yield sequence of photosynthetic O_2 evolution was related to a certain percentage of 'misses' (α; i.e. centers not converted). The possibility of a miss was supposed to be equal for all states $S_{0,1,2,3}$. We propose a new model and a new recurrence law that gives better quantitative agreement with the O_2 yield oscillations observed in Chlorella during a sequence of flashes. We find a better fit with all experimental results by assuming very unequal misses; the misses occur nearly exclusively on S_2 (and also sometimes on S_3). In the simpler case of only one miss on one state, half of S_2 exists as an inactive form S_{2+}^- because it is in apparent equilibrium with pool A. The active form of S_2 is converted to S_3 in a flash and the unchanged inactive form S_{2+}^- explains the miss:

$$S_1 \xrightarrow{h\nu} S_{2+}^- \rightleftharpoons S_2 \xrightarrow{h\nu} S_3$$

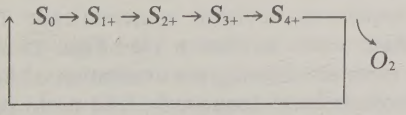
(S_{2+}^- is a transition state between S_1 to S_2 associated with Q^-).

In the dark, the apparent equilibrium constant K_A between pool A and Q (i.e. S_0, S_1 in the dark) is very large; this explains why there is no miss on these states. In light, the experimental value of K_A between pool A and Q (i.e. S_2, S_3 in the light) is 1, and this explains why the misses are large for states S_2, S_3: i.e., $S_{2+}^-/S_2 \simeq 1$ and sometimes $S_{3+}^-/S_3 \sim 0{\cdot}1$.

This new model predicts that the total number of active states $\Sigma S_i = S_0 + S_1 + S_2 + S_3$ is an oscillating function of the flash number. This sum $\sum_i S_i$ is also the number of trapping centers for excitons. As fluorescence is proportional to excitons that are not trapped, our model explains why the fluorescence oscillates as a function of the flash number. We find also that the initial rates of O_2 evolution after $(n-1)$ flashes vs the O_2 yield of the n^{th} flash are not exactly on a straight line, which also favors our model.

INTRODUCTION

Joliot *et al.* (1969) have observed that a sequence of flash yields of O_2 after a dark period shows damped oscillations with a period of 4 flashes. As shown by Kok *et al.* (1970), liberation of O_2 requires the cooperation of four photochemically formed oxidizing equivalents in the individual reaction centers generated by four successive excitations of System II. The model of Kok *et al.* (1970) is a linear four-step mechanism in which a trapping center successively accumulates four (+) charges.



The S_4 state produces O_2 and returns to the ground state S_0.

This model shows that the O_2 yield produced with a flash is equal to the quantity of S_3 present before the flash. After darkness, the first two flash yields are zero, showing that only the state S_0 and S_1 are present in the dark. Forbush *et al.* (1971) have explained the damping of the O_2 yield oscillations by assuming a certain percentage of misses α (centers not converted) even in a very short and saturating flash. Up to now, the reason for these misses was not explained.

We have analyzed on a quantitative basis our experimental flash O_2 yield sequences. We found that very unequal misses give a better fit with the experimental results. In most cases, only one state gives a miss while the other states have no misses. This quite surprising behaviour was observed with nearly all our experimental results. The oscillating

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behaviour of the flash yield sequence is not sufficient to determine the structure of the recurrence relation of the four states after the successive flashes. The model of Forbush *et al.* (1971) is one solution but there was no experimental evaluation of other possible explanations. Results from oxygen and fluorescence measurements afford a means to choose between these other solutions.

MATERIALS AND METHODS

The experiments were performed with *Chlorella pyrenoidosa* Chick Emerson strain grown under standardized conditions. They are grown in a modified medium of Tamiya *et al.* (1953) under low light intensity at a specific growth rate of 1·1 per day. Cultures were gassed with 4 per cent CO_2 in air; the harvested algae were not aerated with CO_2 during experiments. We measured flash yields and rates of O_2 evolution with a rate electrode geometrically identical to that described by Joliot and Joliot (1968). The layer of cells on the platinum electrode was never more than one cell thick; a 25 μm dialysis membrane retained the algae within the small chamber and permitted diffusion of gas and nutrients to the algae from the circulating culture medium. The flashes (3 μs, bandwith measured at 1/3 of the maximum intensity) were produced by a "strobotac" (General Radio Company) and the flash yields of O_2 were observed after a.c. amplification directly on an oscilloscope and recorded photographically in order to suppress any loss of precision. The a.c. amplifier (P.A.R. 113) was directly connected to the polarization circuit in parallel with a resistance of 1 kΩ. This resistance gave the best signal-to-noise ratio. The bandwith was fixed between 3 Hz and 100 Hz to prevent superposition of the tail of one flash and the following flash. The experimental noise was of the order of the oscilloscope spot on the photographs.

The steady state of O_2 was measured with a modulated detecting beam and a 'lock-in' amplifier. The initial rate was measured without a lock-in (but with modulated light) because the rate transients were too fast as compared to the amplifier time constant and the modulating frequency (20 Hz). On the oscilloscope, the signal showed two superimposed transients: the oscillating signal of the O_2 rate at the modulating frequency, and also a slow damped transient. The slow damped transient came from the change of the mean value of oxygen concentration at the beginning of the illumination, because the time of the diffusion of oxygen through the electrode membrane is long ($\simeq$ 0·3 s). This slow transient (over ten periods) prevented us from measuring the initial rate with good precision. Consequently we followed the initial rate after slightly modifying the light modulating disc: we changed the light–dark cycle. The algae were illuminated only one-half period (10 ms) over 8 periods (80 ms), so that the transients observed after each illumination were well separated from each other. With this method the initial rate was recorded photographically with the necessary high precision.

RESULTS

Method of obtaining the best fit between experimental and theoretical flash O_2 yield sequences

Generally in the literature (e.g. Bouges-Bocquet, 1973) the values of S_i from an experimental O_2 yield sequence are calculated from the Forbush *et al.* (1971) model. According to these authors, in any single flash a certain percentage (α) of the centers is not converted (misses); another percentage (β) of the centers may receive two excitations in one flash. Joliot *et al.* (1971) have shown that this factor β can be eliminated if the flash is sufficiently short (3 μs). Since we have used such very short flashes, we shall not take into account this factor β. The Forbush *et al.* recurrence law allows one to compute the concentrations of the states $S_{0\to3}$ after the $(n+1)^{th}$ flash from the ones after the n^{th} flash:

$$\begin{aligned} S_0^{n+1} &= (1-\alpha)S_3^n + \alpha S_0^n \\ S_1^{n+1} &= (1-\alpha)S_0^n + \alpha S_1^n \\ S_2^{n+1} &= (1-\alpha)S_1^n + \alpha S_2^n \\ S_3^{n+1} &= (1-\alpha)S_2^n + \alpha S_3^n. \end{aligned} \tag{1}$$

With the help of a calculator (Hewlett-Packard model 20: the programme was sent to the user's club and can be obtained from this company), the values of the parameters α, S_0^n, S_1^n, S_2^n, S_3^n corresponding to an experimental flash O_2 yield sequence were determined so that the difference between all the theoretical values Y_{th}^n and experimental values Y_{exp}^n was minimum.

This determination was made with the least-squares-approximation method. We minimized the sum of the squared deviations $E = \Sigma_n (Y_{th}^n - Y_{exp}^n)^2$ with respect to the parameters. The recurrence law being linear, Y_{th}^n is a linear function of the initial values $S_{0\to3}^{n=0}$ for a fixed α value (miss). For this reason, minimization of the sum E of the squared deviations is equivalent to resolution of the linear system of four equations $dE/dS_{i=0,1,2,3} = 0$ with respect to the parameters $S_{0\to3}^{n=0}$. Knowing the initial $S_i^{n=0}$ values for a fixed α value, we determined the corresponding value of E; then, by varying α we determined the optimum α value where E is minimum.

Figures 1a and 5 show examples of this curve fitting. The indicated α value, equal for all centers, is the α value corresponding to the minimum square deviation. It was found that the agreement between the experimental and theoretical values of flash sequences deviated systematically for the same flash yields number n (see Figs. 1a and 5).

Our method, allowing the evaluation of S_i and α from experimental sequences, lead us to partially reconsider the generally accepted Forbush *et al.* model where the misses (α) are the same for all 4 states.

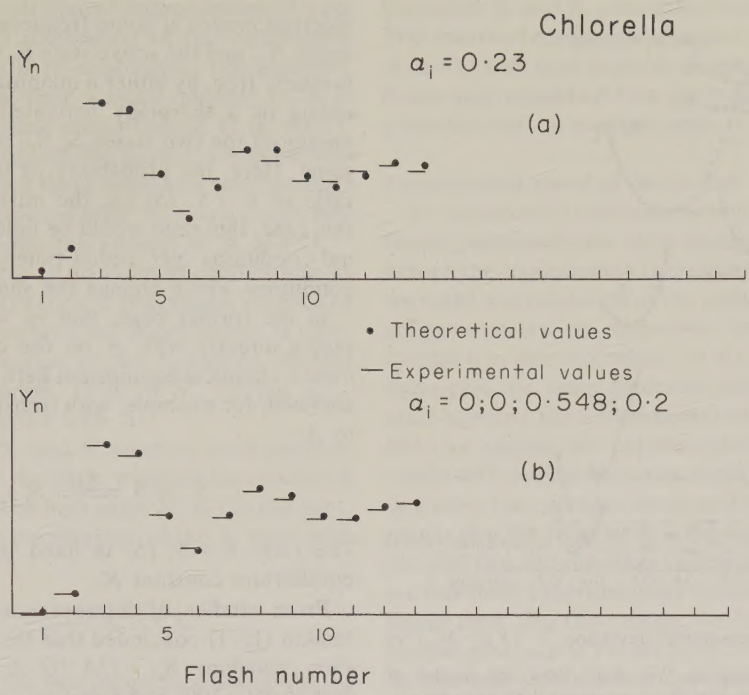

Figure 1. Flash O_2 yield sequence observed with *Chlorella* with saturating flashes after 4 min of darkness. These algae were in darkness a long time (1 h before putting them on the electrode). Spacing between flashes: 300 ms. Dashes are experimental values. Points are theoretical values of S_3 computed either from the Forbush *et al.* (1971) recurrence law or from ours; but the minimum square deviation $\sum_n (Y^n_{th} - Y^n_{ex})^2$ is the same for the two models (Forbush *et al.* model or ours). (a) Best fit with the misses equal for all four states ($\alpha_i = 0{\cdot}23$). (b) Best fit with unequal misses ($\alpha_i = 0$; 0; 0·548; 0·2). The experimental error is of the order of the size of the experimental points.

Experimental determination of the unequal misses

By the method described above, we systematically changed the values of the misses attributed to each state. As shown on Fig. 2, we used a trial and error method. Only one α was varied (α_1 for example), while the others were kept constant. Then, the obtained optimum value of α_1 was kept constant and another α for example, (α_2) varied, and so on. From successive trials it appeared that the difference between the experimental and theoretical Y^n diminishes as three misses approach 0 and one $\alpha \simeq 0{\cdot}5$ (see Figs. 2 and 6). The minimum difference is frequently obtained when three misses are equal to 0 and another close to 0·5. However, for sequences obtained on algae adapted a very long time to darkness, such agreement required the assumption of two unequal misses on two states (Fig. 1b).

Model explaining the misses on one or two states only and determination of a new recurrence law

Misses in each flash, as postulated by Forbush *et al.* (1971), are valid only for non-saturating flashes. In this case the number of excitations are smaller than the number of centers, and during one flash some centers do not receive an excitation and do not react. During a subsequent flash, they may react. But even in super-saturating flashes, where each center can receive an excitation, the damping was not suppressed. It is therefore difficult to understand why the damping persisted even in super-saturating flashes.

It seems natural to suppose that some modifications may occur around the centers S_i which do not react during a flash. State S_i refers to the general state of a center, which is composed of its oxidizing side with i charges (+) and its reducing side Q (Duysens, 1963). Many experiments (Delosme, 1971) have shown that the oxidation level of S influenced the fluoresence which was at first supposed to be a function only of Q. For this reason, we believe that Q and S are intimately connected, and take the notation S_i for the pair S_iQ.

We suppose that some centers stay in an inactive state S^-_{i+} that cannot be excited in order to be transformed into S_{i+1}. The O_2 flash yields sequence is damped only if there is some probability that one center which is inactive during one flash will be active during the following flash. If the same center

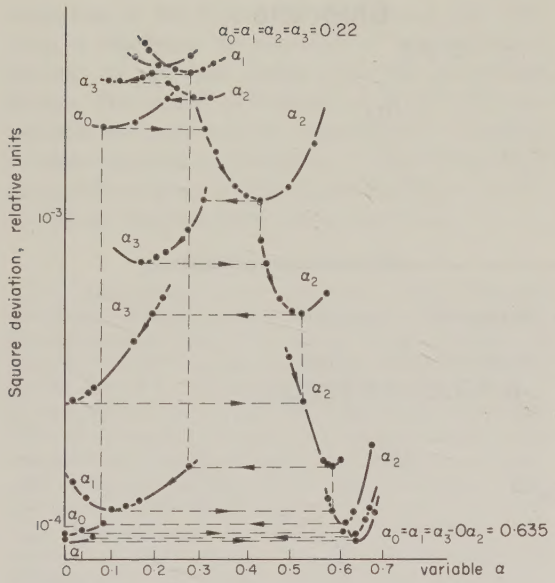

Figure 2. Plot of the squared deviation $\sum_{n=0}^{n=12} (Y^n_{th} - Y^n_{ex})^2$ vs the different varying α_i. We show how the model of unequal misses improves the agreement with experiment. At the top of the figure, we begin with equal misses which is a local minimum when only one miss changes. We must change 2 misses, for example α_1 and α_3, in order to improve the agreement. After these successive variations of α_3, α_2, α_0, and so on, along the arrows, we find the better distribution of the α_i giving the least-squares deviation. The vertical lines show the regions where the α_i do not change.

were always inactive, it would never react and for this reason, we should see only the active centers without damping. For this reason, we must explain the misses with a model describing the state S_i as switching continuously between the active state with some probability r_i and the inactive state S^-_{i+} with the probability $1-r_i$ at some frequency; this switching must be slower than the flash duration (3 μs) and faster than the time between flashes, 0·3 sec. A dynamic equilibrium between the active S_i and inactive S^-_{i+} states must exist. The last one is the state of a photochemical center just after the trapping of an electronic excitation by the active state $i-1$. Afterwards, charge separation occurs giving an active center i and an electron e^-:

$$S^-_{i+} \rightleftharpoons S_i + e^-.$$

The electron can have two fates: it either reacts directly in the center with A ($A + e^- \rightarrow A^-$) or it becomes nearly 'free' and reacts with A in another place. In the latter case, we may imagine that the electron moves at some frequency between the inactive S^-_{i+} and the active state S_i, where the electron is nearly free, by either a quantum-mechanical tunneling or a thermally activated transition if the energy of the two states S_i, S^-_{i+} are not exactly the same. Here, the probability of transition fixes the ratio of $\theta_i = S^-_{i+}/S_i$, i.e. the miss on this state. In this case, this ratio would be independent of external conditions (pH, redox potential) except under conditions which change the shape of centers.

In the former case, that is, when the electron reacts directly with A on the center, there may exist a chemical equilibrium between S_i and S^-_{i+} associated, for example, with transfer of the electron to A:

$$S^-_{i+} + A \overset{K_A}{\rightleftharpoons} S_i + A^-.$$

The ratio $\theta_i = S^-_{i+}/S_i$ is fixed by A^-/A and the equilibrium constant K_A.

From studies of fluorescence on chloroplasts, Malkin (1971) concluded that the apparent equilibrium constant $K_A = QA^-/Q^-A$ is very high in darkness (>100) and equal to 1 in light. The existence of a low apparent equilibrium constant between the pool A and Q in light was previously shown by A. Joliot (1968) with O_2 measurements which more precisely gave S_3 instead of Q. If we suppose that the misses come from a chemical equilibrium, their values must vary with the external conditions. Bouges-Bocquet (1973) showed that the ratio S_0/S_1 and also the damping of the sequence depended upon the redox potential of the medium: in particular the damping increased when A was reduced, but not as much as the ratio S_0/S_1. Recently, Radmer and Kok (1973) have studied the flash yield pattern of chloroplasts as a function of the reduced state A^- of A. (In the notation of Kok and Joliot, the A_2 pool corresponds to our A). They observed that the damping of the sequence increased when reduced A_2 increased. They also noted that, as long as Q and A_2 are reduced, no oscillation of the flash yield was observed. In Chlorella, the restoration kinetics of A_2, by the system I is very fast, so that we were unable to make similar observations. The results of Radmer and Kok (1973) are in agreement with the concept that an apparent equilibrium constant between the states S_2 or S_3 and A_2 explains the origin of misses. Proof of the establishment of an apparent equilibrium between Q and A equal to 1 is also given by Forbush and Kok (1968): with a flash, Q is completely reduced and at the end of the flash no equilibrium exists. In the dark period following the

flash, however, in half time of approximately 0·6 ms (with chloroplasts) restoration of the equilibrium between A and Q is re-established. For example, after the first flash, only a small quantity of A is reduced, but the same proportion of Q is reoxidized. In order to explain the misses, we must suppose that in darkness there remains a small amount of A^-. This is consistent with the usual result that far-red light increases the amount of A and Q.

In the dark, there is no defined equilibrium, as shown by Malkin (1971); the restoration kinetics of Q in the dark observed by fluorescence is attributed to recombination of the remaining charges (−) and (+) (Bennoun, 1970) of S_{i+}^- (giving $S_{(i-1)}$) which has not reacted with A.

The misses on S_0 and S_1 must be small because K_A is very high in the dark, whereas the misses on S_2 and (or) S_3 must be high since K_A is 1 in the light.

Considering the equilibrium of the S_i state with S_{i+}^-:

$$S_{(i-1)+} \xrightarrow{h\nu} S_{i+}^- \rightleftharpoons S_{i+}.$$

We determined the new recurrence law giving the S_i^{n+1} values after a flash $n+1$ as a function of the S_i^n values after the preceding flash n. We suppose that in a sequence of O_2 flash yields, the misses are due to the inactive center S_{i+}^- that have not reacted. Before the flash, the equilibrium gives: $\alpha_i = S_{i+}^-/S_i + S_{i+}^-$ and $S_i = (1-\alpha_i)(S_i + S_{i+}^-)$. During the flash $n+1$, the state S_{i-1}^n becomes an active state i and the state $S_{i+}^{-n} = \theta_i S_i^n = [a_i/(1-a_i)]S_i^n$ remains an inactive state i. After the flash, the equilibrium restores the ratio α_i, keeping constant the total amount of the two states i, inactive and active, equal to $S_{i-1}^n + S_{i+}^{-n}$, so that: $S_i^{n+1} = (1-\alpha_i)(S_{i-1}^n + S_{i+}^{-n})$ and the recurrence law is:

$$S_i^{n+1} = (1-\alpha_i)S_{i-1}^n + \alpha_i S_i^n. \qquad (2)$$

(For $i = 0$, we have $i - 1 \equiv 3$ according to the cyclic permutation of the four states.) This recurrence law is different from that used by Forbush *et al.* For unequal misses, the recurrence law of Forbush *et al.* (formula 1) is:

$$S_i^{n+1} = (1-\alpha_{(i-1)})S_{(i-1)}^n + \alpha_i S_i^n. \qquad (3)$$

With our new recurrence law, and with the same values of misses, we obtained the same minimum square difference between the experimental and theoretical values as with the law of Forbush *et al.* (1971). The new recurrence law does not change this minimum difference (see Appendix A) but only the initial S_0 and S_1 concentrations (Figs. 5 and 6). The observed equilibrium constant $K_A \simeq 1$ between A and Q with light explains qualitatively why there is one miss equal to 0·5 on one state and sometimes a smaller one on another state.

Experimental proof of the model

In numerous experiments, only one miss was found, particularly for algae illuminated a long time before the experiments (example in Fig. 6). When we make a permutation of the unique miss α_i on the different states, as shown in Appendix A, both models (i.e. that of Forbush *et al.*, 1971, or ours) always give the same minimum mean square difference between the experimental and theoretical Y_n. We can explain the experimental oscillating flash yields with exactly the same accuracy with the unique miss, for example, on S_0 or S_1 or S_2 or S_3. This means that the value of S_3 is the same for these four possibilities, but the other values of S_0, S_1, S_2 which are unknown experimentally oscillate with quite different laws. For this reason, we do not know if the unique miss $\alpha_i = 0{\cdot}5$ is on S_2 or S_3 or even on S_0 or S_1 with only the experimental O_2 yield sequence. At this time we do not know how to measure the concentration of S_0, S_1 or S_2. But experimentally we can obtain some information about the sum of states $\sum_i S_i$. One way to obtain this information is by analysis of fluorescence data described in the literature (Delosme, 1971; Joliot *et al.*, 1973). It is clear that the concentration of the sum

$$\sum_i S_i = S_0 + S_1 + S_2 + S_3$$

as a function of the flash number must vary in our model but not in that of Forbush *et al.* (1971). Contrary to the Forbush *et al.* model, where, as a function of number of flashes $\sum_i S_i = C^{te}$, in our model only the sum $\sum_i (S_i + S_{i+}^-)$ is constant. The following results will be interpreted according to this difference.

But first we have to present the different explanations of the non-linear relation between the flash yield of O_2 (i.e. S_{3st}) and the steady-state rate of O_2 which is used to calculate the O_2 rate and the fluorescence as a function of ΣS_i.

a. Exciton transfer process in the photosynthetic unit

Light produces excitations (or excitons) of concentration ϵ at a rate $k_{II}\, aI$ (I: number of light quanta absorbed by the photosynthetic system, a: proportion of quanta absorbed by system II, k_{II}

maximum quantum yield of system II). These excitations have a life time k_T^{-1} which decreases their number at the rate-$k_T\epsilon$ proportional to the fluorescence of system II. The excitations are trapped by the photosynthetic trapping centers with a rate $-\epsilon(p_0S_0 + p_1S_1 + p_2S_2 + p_3S_3)$, where the factors p_i are rate constants or probabilities of trapping by the centers in each state *i*. At limiting light intensity the rate of photon adsorption, i.e. the rate of excitation production, is limiting, and the concentration of excitations is stationary.

$$\frac{d\epsilon}{dt} = k_{II}aI - \epsilon \sum_i p_iS_i - k_T\epsilon = 0. \quad (4)$$

(See Lavergne and Lavorel, 1972.)
This equation yields ϵ (proportional to the fluorescence $k_F\epsilon$). The rate of photosynthesis is:

$$v_{O_2} = \frac{1}{4} \times \frac{k_{II}aIp_3S_3}{k_T + \sum_i p_iS_i} \quad (5)$$

($\times 1/4$ is used because v_{O_2} is measured in O_2 molecular units; there are four photoacts to produce an O_2 molecule). This relation gives a simple explanation of the non-linear relation between v_{O_2} and S_3 during a continuous stationary illumination, as first observed by Joliot and Joliot (1964). In this case, the rates of the photoreactions of the four states are equal:

$$p_0S_0 = p_1S_1 = p_2S_2 = p_3S_3 \quad (6)$$

and

$$v_{st} = \frac{1}{4} \frac{k_{II}aIp_3S_3}{(k_T + 4p_3S_3)} \quad (7)$$

as steady-state rate.

The conclusions from this model are identical with those found by Joliot and Joliot (1964), who assumed successive transfer of excitations between inactive centers. The observed non-linear relation of Joliot (1964) $v_{O_2} = k'_{II}\, aI\, [e/(1 - p + ep)]$ gives in this model the ratio $k_T/4p_3S_m = (1 - p)/p$. The term S_m is the maximum available value of S_3 when the relative concentration of active centers $e = S_3/S_m$ is 1; (p is Joliot's probability factor of energy transfer between system II units).

Lavorel (1967) has used a model of limiting diffusion of excitations with random walk. The mathematically more simple chemical model described above is true in the limit of very fast diffusion (Lavergne and Lavorel, 1972). All of the sophisticated models of Joliot and Lavorel (1967), like the chemical one, give a non-linear relation between v_{O_2} and S_3 very close to the formulae (2) and (4) because in all these models there is a competition between the trapping rate of the active centers and the lifetime of traveling excitations. For this reason it is, at this time, impossible to distinguish between them experimentally. One direct proof of this statement is the decrease of the lifetime of fluorescence during photosynthesis of the order of $(k_T + \sum_i p_iS_i)^{-1}$ compared to the case when photosynthesis is inhibited, k_T^{-1}, as observed by Mar *et al.* (1972) and Moya (1973).

b. Initial rate v_i *after* n-1 *flashes as a function of the yield of the flash* n:Y_n

As shown in Fig. 3, the different points corresponding to the initial rate after $(n - 1)$ flashes as a function of Y_n deviate from the straight line corresponding to the slope v_{st}/Y_{st} in steady state (normalized to 1 on abscissa and ordinate). Our experiment of initial rates were made with 700 nm light (6 Wm^{-2}). The flash sequence Y_n obtained with

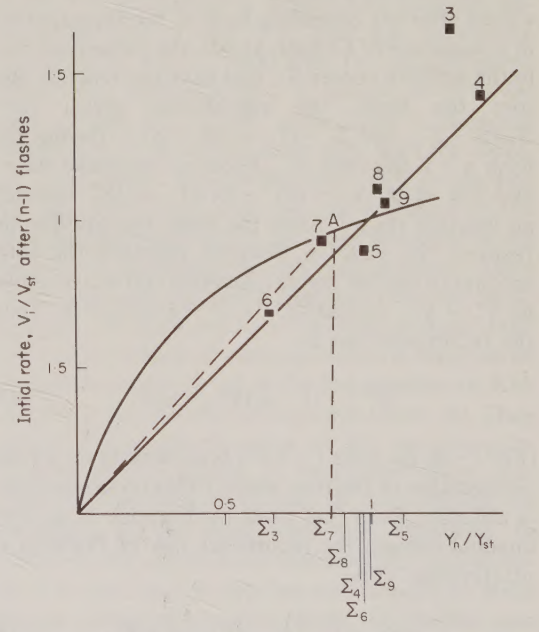

Figure 3. Plot of the initial rate v_i with 700 nm light (6 W m^{-2}, band-width 6·6 nm) observed after flash $(n - 1)$ vs the yield of the flash (n). Spacing between flashes, and between flash $(n - 1)$ and admission of 700 nm light beam: 300 ms. The slopes joining the origin to the experimental points cut the non-linear curve (steady-state rate v_{st} as a function of steady-state O_2 yield $S_{3_{st}}$) at a point *A*. Abscissa of *A* is proportional to the relative $\Sigma_i \beta_iS_i$ values after flash *n*.

the same algae sample is represented in Figs. 5 or 6. This observation is not reported in the literature (Kok *et al.*, 1970) because in general the methods used (with "lock-in" amplifier) do not give good precision. It is the systematic deviations from the straight line as a function of the flash number (the points describe a flat spiral around the equilibrium point on Fig. 3) which lead us to improve the precision of measurements.

As indicated in formula (5), in non-steady-state rate, when S_3 varies, v_{O_2} is on a straight line if $\sum_i p_i S_i$ is constant. In our experiment this was not the case (Fig. 3). Consequently, our experiment means that the sum $\sum_i p_i S_i$ varies as a function of the flash number. During the same experiment the non-linear curve $v_{O_2st} = f(S_{3st})$ was determined (S_{3st} was varied by adding a continuous 650 nm light to the modulated 700 nm light). In steady-state rate, from Eq. 6 we can calculate that $\sum_i p_i S_i = 4p_3 S_{3st}$. For a non-steady-state rate and for a fixed value of $\sum_i p_i S_i$, the initial rate v_i is on the straight line of slope v_i/S_3 as S_3 varies (see Fig. 3). When S_3 is equal to S_{3st} of the steady-state rate, the straight line of the slope v_i/S_3 intercepts the non-linear curve at a point where $4p_3 S_3 st = \sum p_i S_i$. For this reason, the abscissa of this point S_{3st} gives directly the value of $(1/4p_3) \sum_i p_i S_i$, i.e. a relative value of the sum $\sum_i p_i S_i$. Therefore the slope $v_{i_{n-1}}/Y_n$ cuts the non-linear curve at points of the abscissa proportional to the different relative $\sum_i p_i S_i$ values at the n^{th} flash (see Fig. 3). On Fig. 7*a*, we have plotted these values $\sum_i p_i S_i$ as a function of the flash number.

The sum $\sum_i p_i S_i$ may vary if the relative probabilities of trapping by the centers $\beta_i = p_i / \sum_i p_i$ are not the same. The relative values of these probabilities β_i can be determined from the oscillation O_2 flash yield sequence obtained just after a continuous illumination. As shown in Fig. 4, after a continuous light (even if strong) an oscillating sequence was observed with *Chlorella* (but not with chloroplasts). This result is not reported in the literature because the continuous illumination must be stopped at the first flash. Otherwise the continuous light suppresses the oscillations by equalizing the rates of Eq. 6 between two flashes. We have studied these oscillations as a function of the light intensity and found that they remain even in saturating light (superimposed on the restoration of pool A in this case). From Eq. 6, the corresponding relative values of p_i (β_i) can be determined if the steady-state values of $S_{0\to3}$ are known during illumination.

From an experimental sequence (after illumination or after darkness), the $S_{0\to3}$ values were determined using either the approach of Forbush *et al.*, or ours, as shown in Fig. 5 and 6. The sums $\sum_i p_i S_i$ as a function of the flash number were then calculated and these sums are represented in Fig. 7. In the Forbush *et al.* model, $\sum_i S_i$ being constant, the only oscillations of the curve $\sum_i \beta_i S_i$ (Fig. 7*f*) are due to the unequal values of β_i. These oscillations look like those experimentally obtained (Fig. 7*a*) but their amplitudes are much smaller. Consequently in our model the oscillations of $\sum_i p_i S_i$ come primarily from that of $\sum_i S_i$ because the values for β_i are nearly identical. In Fig. 7*b*, *c*, *d*, *e*, we have represented the sum $\sum_i \beta_i S_i$ computed from our model when the unique miss is successively on one state α_i while the other states have no misses. Only when α_2 was the unique miss (case *b* of Fig. 7) was good agreement found between the theoretical values of

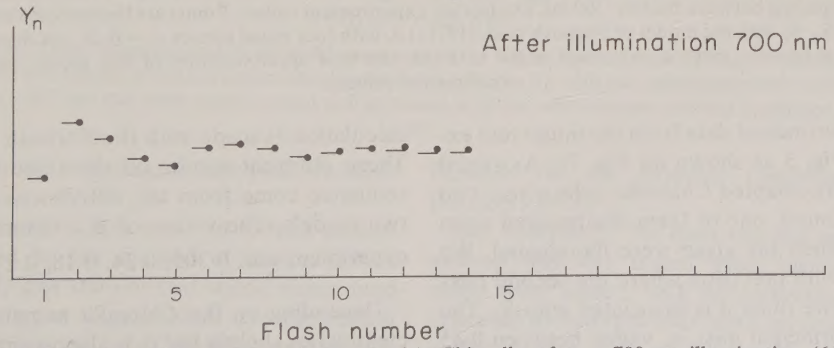

Figure 4. Flash O_2 yield sequence observed with *Chlorella* after a 700 nm illumination (6 W m^{-2}, band-width 6·6 nm). The 700 nm light is cut when the first flash of the sequence is given. Time between flashes: 300 ms. Similar oscillations of the flash yield sequence are observed after illumination with other wavelengths.

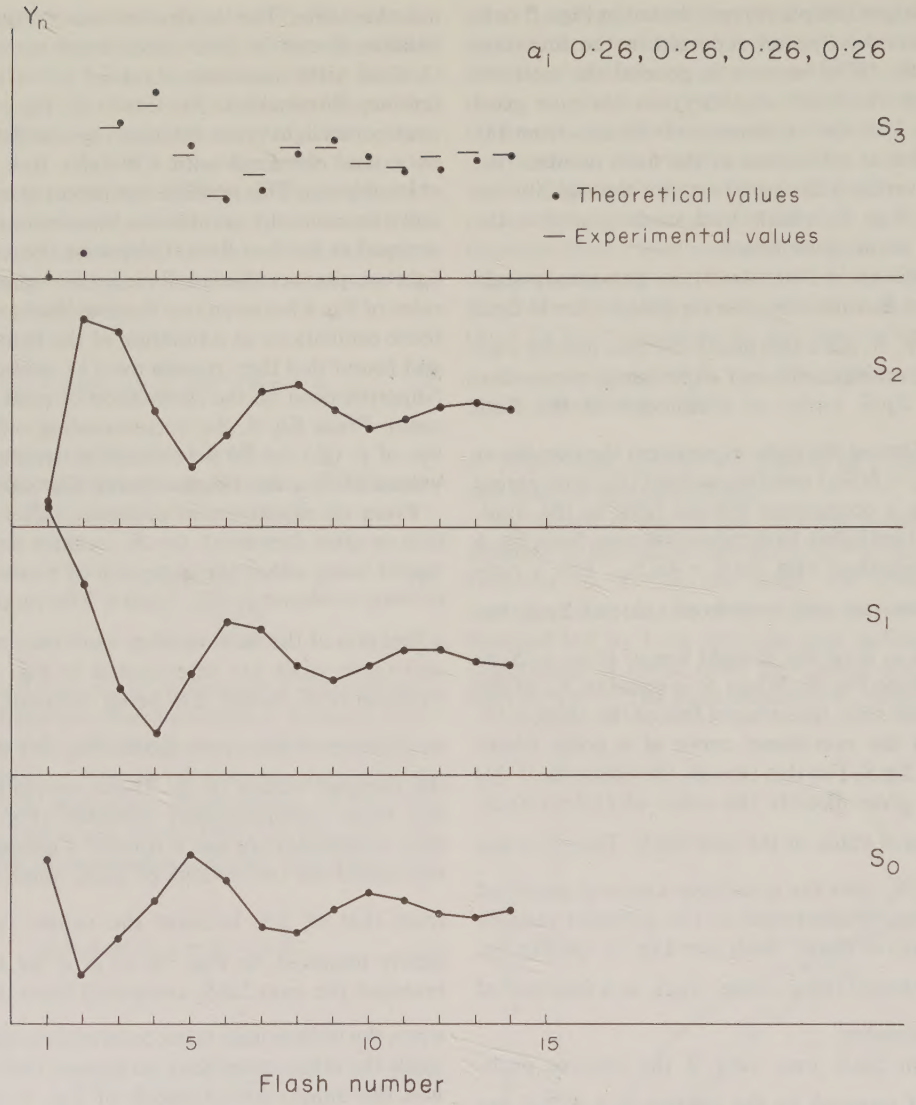

Figure 5. Flash O_2 yield sequence after 6 min darkness observed with the same sample of *Chlorella* as for Fig. 3; spacing between flashes: 300 ms. Dashes are experimental values. Points are theoretical values of S_0, S_1, S_2, S_3 with the model of Forbush *et al.* (1971) i.e. with four equal misses $\alpha_i = 0{\cdot}26$, obtained with the least-squares method explained in the text (i.e. the best approximation of this model with the experimental values).

$\Sigma\beta_i S_i$ and experimental data from the initial rate experiment of Fig. 3 as shown on Fig. 7*a*. As stated before, for dark-adapted *Chlorella* cells where two misses were found, one of them disappeared upon illumination when the algae were illuminated. We do not know with precision where the second miss occurred, but we think it is associated with S_3. The value of the principal miss α_2 varies between 0·45 and 0·7, and α_3 varies from 0·3 to 0. By comparing Figs. 5 and 6, one can see that in our new model the ratio S_1/S_0 is high; $\simeq 3{\cdot}1$ instead of 1·6 when the calculation is made with the Forbush *et al.* model. These different results on the same experimental sequence come from the differences between the two models. The values of $\beta_{0\to 3}$ determined in this experiment are: 0·305, 0·24, 0·18, 0·275 if $\sum_i \beta_i = 1$.

Depending on the *Chlorella* sample, this coefficient varies slightly but β_2 is always smaller than the other β_i. In other experiments we found that neither DCMU [3 (3-4, dichlorophenyl)-1, 1-dimethylurea] nor CCCP [carbonyl cyanide *m*-chlorophenyl-

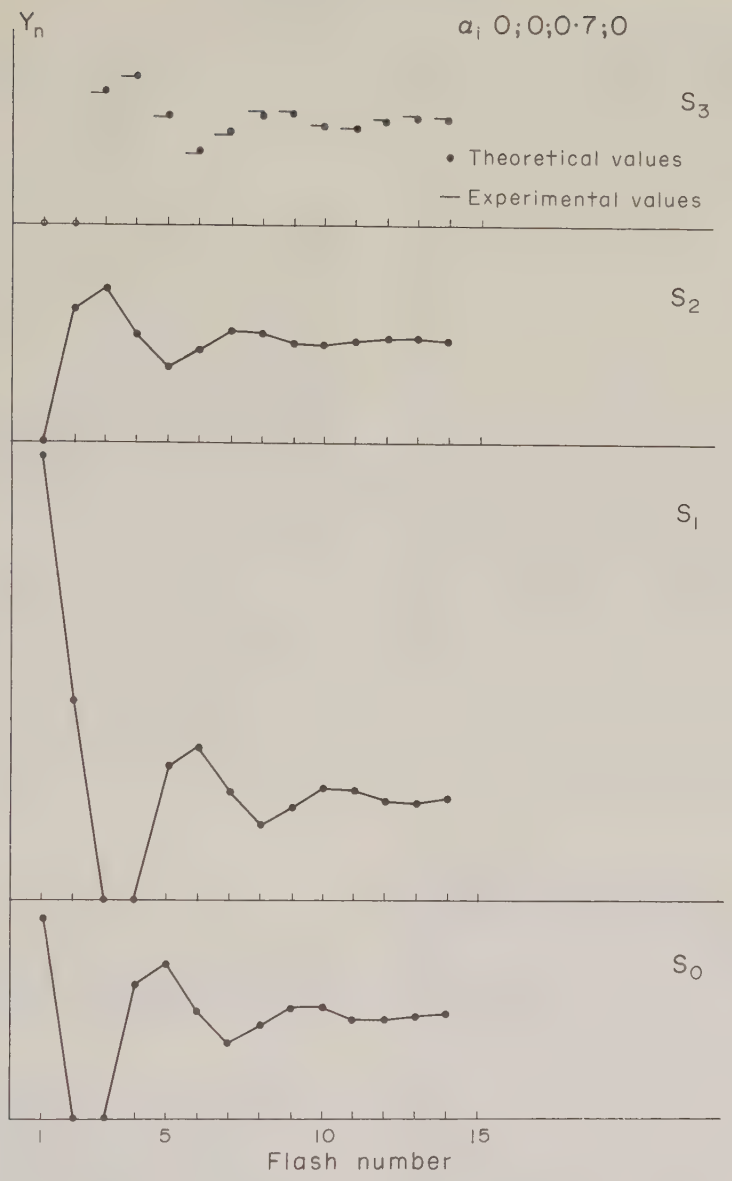

Figure 6. Same experimental flash yield sequence after 6 min darkness as in Fig. 5. Dashes are experimental values. Points are theoretical values of S_0, S_1, S_2, S_3, with the model presented in the text with $\alpha_2 = 0{\cdot}7$ and the other misses equal to 0 obtained with the least-squares method explained in the text.

hydrazone] (for 1/2 inhibition of flash yield) changed the value of α_2.

The same analysis was used on lettuce chloroplasts obtained by the method of Avron (1960). Misses on only one state or two states were found and the fit between experimental and theoretical sequences was as good as in *Chlorella* (especially in presence of N_2, because a rapid O_2 photoabsorption simultaneous with the O_2 yield alters the measurement). The dark concentration of S_0 and S_1 strongly depends on the external conditions (Bouges-Bocquet, 1973).

Our analysis by the least-squares approximation method was also tested when misses of known origin were added; with nonsaturating flashes we expected to find that four equal misses were added to the unique miss of 0·6 found with saturating flashes. As expected, in this example, the best fitting was obtained with the following misses on the four states: 0·1, 0·1, 0·7, 0·1.

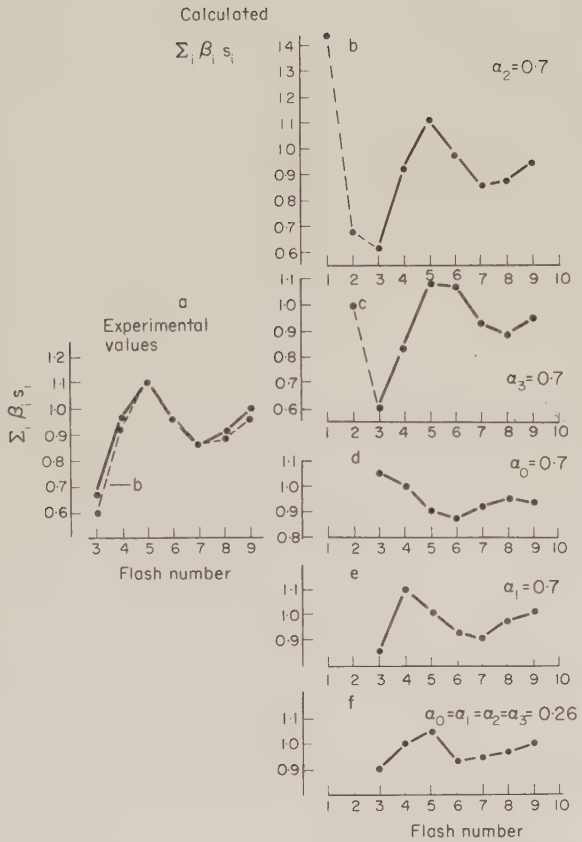

Figure 7. a: Experimental values of $\Sigma_i \beta_i S_i$ obtained from Fig. 3 vs the flash number *n*. *b*, *c*, *d*, *e*, *f*: Calculated values of $\Sigma_i \beta_i S_i$ depending on the values of misses α_i as shown. *f*: all misses equal; *b*, *c*, *d*, *e*, only one miss on one state, respectively on S_2, S_3, S_0, S_1. The experimental values are in close agreement with one miss on S_2.

Simple recurrence law due to the unique miss on S_2. As said before, two misses or one miss can be found when we analyse the O_2 yield sequence. In the case of only one miss, we have the following distribution of S'_i after the flash as a function of the S_i before the flash:

$$S_0 \rightarrow S_1 = S'_1$$
$$S_1 \rightarrow \alpha_2 S_2 + (1-\alpha_2) S_1 = S'_2$$
$$S_2 \rightarrow S_3 = S'_3$$
$$S_3 \rightarrow S_0 = S'_0.$$

The flash O_2 yield Y is equal to the abundance of S_3.

The yield of the flash $(n+4)^{th}$ is:

$$Y^{n+4} = S_2{}^{n+3} = \alpha_2 S_2{}^{n+2} + (1-\alpha_2) S_1{}^{n+2},$$

so that

$$Y^{n+4} = \alpha_2 Y^{n+3} + (1-\alpha_2) Y^n,$$

and

$$Y^{n+4} - Y^n = \alpha_2 (Y^{n-3} - Y^n). \qquad (8)$$

When $Y^{n+4} - Y^n$ were plotted vs $Y^{n+3} - Y^n$ (ordinate and abscissa, respectively) we obtain points on a straight line, whose slope gives directly the α_2 value shown in Fig. 8. This property can be used graphically to determine the α_2 value (if the flash is short enough). When there are two misses (after a long dark period) there is some deviation from this straight line. This simple graphical rule is useful for a fast analysis of the experimental data.

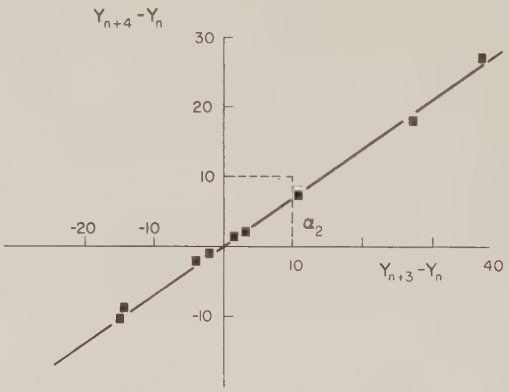

Figure 8. With the experimental flash yield sequence of Fig. 5 or Fig. 6 is plotted $Y_{n+4} - Y_n$ vs $Y_{n+3} - Y_n$. The slope of the straight line joining the different points is equal to α_2 when there is only one miss.

Fluorescence

Fluorescence oscillation as a function of the number n *of flashes.* Figure 7b shows the variations of the total numbers of states as a function of the number of flashes *n* in our model. Depending of the ratio S_1/S_0 and the number of misses, these variations can change slightly with the alga sample (in particular for the 2nd and 3rd flash).

Our model explains naturally why the fluorescence in low light is an oscillating function of the number *n* of flashes (Delosme, 1971, Fig. 3b).

The number of traps for excitons is equal to the total number $\Sigma_i S_i$ of active states. The fluorescence, proportional to the number of excitons which are not trapped, must vary as the inverse of the sum $\Sigma_i S_i$.

The formula (1) gives the number of excitons

with weak light:

$$\epsilon = \frac{k_{II} a I}{k_T + \sum_i p_i S_i} \tag{9}$$

and the fluorescence

$$\phi = k_F \epsilon = \frac{k_F k_{II} a I}{k_T + \sum_i p_i S_i} \tag{10}$$

k_F/k_T is the proportion of excitons decaying by fluorescence when the photosynthesis rate is zero, i.e., when $\sum_i p_i S_i = 0$. Because the total number of states $\sum_i p_i S_i$ is an oscillating function of the number n of flashes in our model (and not in the Forbush *et al.* model, where $\sum_i S_i$ = constant), the fluorescence must reflect the same oscillating function as the slope of the straight line connecting the experimental points with the origin of the coordinates in Fig. 3 (the complementary oscillations of those shown on Fig. 7b). If we use our results to explain the results of Delosme (1971) (Fig. 3b), we find a qualitative but not quantitative agreement (see Fig. 9). This

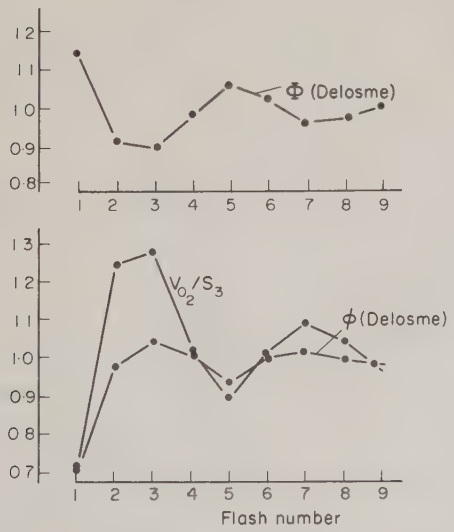

Figure 9. As a function of flash number n is shown: + ϕ, fluorescence emitted during a strong flash in *Chlorella* [from Delosme (1971) Fig. 3a]; + φ, fluorescence emitted during a weak detecting flash [from Delosme (1971) Fig. 3b]; + v_{o_2}/S_3 i.e. the slope of the straight line joining the origin to the experimental points of Fig. 3. (The oscillations of v_{o_2}/S_3 would be the same as the ones of the low-level fluorescence from formula (7) if we take into account the variation of fluorescence yield ϕ with state S_2^-.

may be due to the difference in the quantitative behaviour of algae of different origins. The original model of Forbush *et al.* cannot explain the oscillating values of $\sum_i p_i S_i$ if the value of p_i are nearly equal (as is the case here) because the total amount of $\sum_i S_i$ is constant in that model. For this reason, Joliot *et al.* (1971) and Delosme (1971) observed a correlation between the oscillation of the fluorescence yields after n-1 flashes and $S_2 + S_3$ using the Forbush *et al.* model.

It seems surprising that all the different kinds of fluorescence measurements oscillate like $\sum_i p_i S_i$ or $1 - \sum_i p_i S_i$ in our model. For example, Fig. 3*a* of Delosme (1971) shows that the fluorescence emitted during a strong flash ϕ (also represented in Fig. 9) oscillates about ± 10 per cent with the number n of the flash. In our model, this fluorescence must not oscillate because all the Q are in the reduced form Q^- (or the S state in the excited form). One simple way to explain this is to suppose that the different inactive S_{i+}^- states are not rigorously inactive and that for some of them (especially S_{2+}^- and perhaps S_{3+}^-), there remains some probability of trapping (more precisely, of deactivation) of the excitation without photosynthesis, giving a variable level of fluorescence during the flash. This hypothesis could explain the discrepancies between the fluorescence oscillations and the oscillations of the slope v_{O_2}/S_3 after flashes (see Fig. 9).

To interpret the fluorescence oscillations, Joliot and Joliot (1971) have proposed a model of two hypothetical quenchers (Q_1, Q_2) with very different quenching efficiency: Q_1 is associated with S_0 and S_1, with high quenching efficiency (low fluorescence), and Q_2, with a low quenching efficiency (high fluorescence), is associated with S_2 and S_3. In our model, Q_1, corresponding to $\sum_i S_i$, has a high quenching efficiency; Q_2 equal to $\sum_i S_{i+}^-$ with small quenching increases the fluorescence level. We do not need an additional arbitrary hypothesis to explain why there are fluorescence oscillations.

Fluorescence in presence of DCMU, and at low temperature. The fluorescence induction with DCMU can be explained with a model of this kind, as proposed by Doschek and Kok (1972). $Q \xrightarrow{h\nu} Q \xrightarrow{h\nu} Q^{2-}$ or, alternatively, $Q \xrightarrow{h\nu} Q^- \xrightarrow{R \to R^-} Q \xrightarrow{h\nu} Q^-$ Q^- being restored by a secondary acceptor R of equal abundance.

We think that, in our model, Q can be assumed to

be identical with the state S_1, the state in darkness in chloroplasts, Q^- with S_{2+}^-, R and R^- with A and A^-. The second state of Q would be S_2 and the DCMU blocks the reaction $S_{3+}^- \rightarrow S_3$. This gives the quite similar model:

$$S_{1+} \xrightarrow{h\nu} S_{2+}^- \overset{AA}{\rightleftharpoons} S_{2+} \xrightarrow{h\nu} S_{3+}^-/\text{DCMU blocking.}$$

This model predicts that the half time of the reaction between S_{2+}^- and A is of order of 0·6 ms (Forbush and Kok, 1968)].

The features of the fluorescence rise curve with DCMU observed by Doschek and Kok (1972) are in agreement with this model: in particular a two-quantum process occurs either after far-red light before DCMU, or a very long dark period after DCMU, i.e. in a fully restored system. The one-quantum induction curve, observed after a 30·s dark period following a blue illumination bringing the fluorescence to F_{max}, is well explained by the non-linear formula used above for v_{O_2} during an illumination (Joliot, 1964), because during a 30 s of darkness only the deactivation of S_{3+}^- occurs. The deactivation of S_2 takes more time in comparison to that of S_{3+}^-. The fluorescence kinetics in that condition depend on two variables S_1 and S_2, instead of Q. This explains why the fluorescence rise after partial restoration is faster than after complete restoration. Figure 10 shows a theoretical evaluation

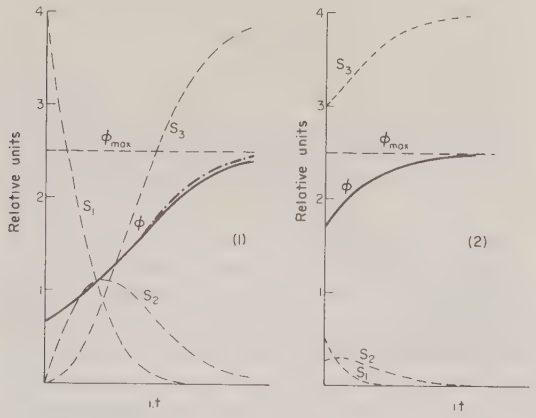

Figure 10. Fluorescence kinetics φ with DCMU calculated with equations in text: S_1 gives S_{2+}^- in equilibrium with S_2 with the same ratio $\theta_2 = S_{2+}/S_2 = 1$ as without DCMU, and S_2 gives S_{3+}^- blocked by DCMU. We assume that the relative probability of excitations trapping $p_1 = 0{\cdot}2785$ and $p_2 = 0{\cdot}21$ are the same as without DCMU. The intrinsic lifetime of fluorescence k_F^{-1} is in relative units $k_F = 0{\cdot}4$ for deactivation and fluorescence with DCMU. After partial dark restoration, we suppose that $S_{3+}^- = 3$, $S_2 = S_{2+}^- = 0{\cdot}25$ and $S_1 = 0{\cdot}5$, so that the kinetics are faster for the same level of fluorescence than for the whole induction curve, where $S_1 = 4$, $S_2 = S_{2+}^- = S_3 = 0$ at the beginning.

of this effect (Lavorel, 1972, with his model of three states, gives nearly the same mathematical explanation).

With DCMU, after one or two flashes, the fluorescence level does not reach its maximum level. Therefore, one flash is not sufficient to reduce all Q in the presence of DCMU (i.e. the equivalents of S_1 or S_2) because, as stated above, there always remains some inactive form of S_{2+}^- in equilibrium with S_2. Before the first flash we have initially S_1^0; after the first flash, we have $S_2^0 = S_1^0\,(1-\alpha_2)$, and $S_{2+}^{-1} = S_1^0\alpha_2$. After the n^{th} flash we have: $S_1^n = 0$, and $S_{3+}^{-n} = S_2^{n-1}$; so $S_2^n = S_1^0(1-\alpha_2)\alpha_2^{n-1}$. The fluorescence varies like the inverse of $\sum_i S_i^n$ (formula 10):

$$\sum_i S_i^n = S_2^n = (1-\alpha_2)\alpha_2^{n-1}S_i^0. \qquad (11)$$

Hence we expect that in presence of DCMU the fluorescence level after n flashes increases in geometrical progression. All the features of this model are in qualitative agreement with the experimental results (Delosme, personal communication). Because the negative charge (−) cannot react with A in the presence of DCMU, the electronic charges e^- accumulate, and the associated electric field probably blocks the reaction $S_{3+}^- \rightarrow S_3$. For this reason we expect that the ratio $S_{2+}^-/(S_{2+}^- + S_2) = \alpha_2$ increases as a function of the number n of flashes in order to block the reaction. It follows that the fluorescence must increase faster, with n than predicted with a constant α_2 value.

In our model, the Q_F quenching introduced by Joliot and Joliot (1973) (variation of fluorescence with 1 flash applied at low temperature) is connected to the value of $\sum_i p_i S_i$, which is naturally the same oscillating function of the number of preilluminating flashes before freezing. This is evident if the reaction of the states S_i with A is blocked, and the negative charges e^- accumulate as for DCMU. The qualitative agreement is good (see Fig. 6 of Joliot and Joliot, 1973). With our model the quenching component Q_S of fluorescence which cannot be removed by a flash is directly the amount of S_{2+}^- state in equilibrium with S_2. This equilibrium persists at low temperature, because it is only a large charge separation. The experimental oscillation of Q_S with the preilluminating flash number is in quite good agreement with those of S_2 (see Fig. 6 of Joliot, 1973) even if, as in *Chlorella*, the concentrations of the various states are not identical to those in chloroplasts.

All these experimental observations are at least

in qualitative agreement with this model, but more quantitative detailed fluorescence studies are necessary to check the validity of our very simple assumptions.

CONCLUSION

The observed fluorescence oscillations as a function of the flash number (Delosme, 1971) show that, during charge separation on one center, the charges (+) and (−) interact. We cannot speak of one quencher Q^- and of four states S_+ separately: the quenching efficiency of Q^- is a function of the four states of charge (+). As Q^- prevents the accumulation of an additional charge (+) on S_{i+}, one could say that the interaction leads to one inactive total state S^-_{i+}.

If we suppose that the miss occurs on S_2 (in the simpler case), from an equilibrium between an active state S_2 and an inactive state S^-_{2+}, we find a good agreement with experiments giving different kinds of oscillations: in particular the initial O_2 rate after $(n-1)$ flashes as a function of Y^n, and the fluorescence as a function of flash number. Both oscillate like the total amount $\sum_i S_i$ of active states. Contrary to previous models, our model explains both the misses, i.e. the damping of the O_2 yield seqeunce and the fluorescence oscillations without too detailed and numerous assumptions. We have concluded that the low value of the equilibrium constant between S_2 and A, and sometimes S_3 and A, is related to the misses because the ratio A^-/A has an influence on the misses, as shown by Radmer and Kok (1973). The equilibrium may be an apparent equilibrium; we may suppose a thermal activated transfer between an active state and an inactive state for S_2 and S_3 explaining that some charge separation occurs at low temperature. We believe that our model provides a way to interpret, within a unique model, all the oscillations observed with different types of measurement as a function of the flash number.

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APPENDIX
MATHEMATICAL REMARKS ON THE LINEAR RECURRENCE LAW WITH FOUR STATES

The general form of a linear recurrence law with four states $\mathbf{S}$ is of the form:

$$\mathbf{S}^{(n-1)} = \mathbf{A}\mathbf{S}^{(n)} \text{ i.e. } S_i^{(n+1)} = \sum_j a_i^j S_j^{(n)}$$

where $S^{(n)}$ is the vector of the concentration of the four states $S^{(n)}_{0\to3}$ and A is the 4 by 4 matrix defining the recurrence law (for example the two simple recurrence laws used in the text). We want to show that many different laws of this kind can equally well explain the same sequence of oscillating flashes with identical accuracy. It suffices that all these laws are defined by matrices A with the same four eigenvalues $\lambda_{0\to3}$. A matrix A can be written:

$$\mathbf{A} = \mathbf{U}\begin{bmatrix}\lambda_0 & 0 & 0 & 0\\ 0 & \lambda_1 & 0 & 0\\ 0 & 0 & \lambda_2 & 0\\ 0 & 0 & 0 & \lambda_3\end{bmatrix}\mathbf{U}^{-1}$$

where U is the matrix defining the transformation of the unit vector of $\mathbf{S}^{(n)}$ into the eigenvectors of the matrix A.

With this remark, we can write:

$$\mathbf{S}^{(n)} = \mathbf{A}^n\mathbf{S}^0 = \mathbf{U}\begin{bmatrix}\lambda_0{}^n & 0 & 0 & 0\\ 0 & \lambda_1{}^n & 0 & 0\\ 0 & 0 & \lambda_2{}^n & 0\\ 0 & 0 & 0 & \lambda_3{}^n\end{bmatrix}\mathbf{U}^{-1}\mathbf{S}^0.$$

The flash n gives the concentration of $S_3^{(n)}$; hence: $S_3^{(n)} = a_0\lambda_0^n + a_1\lambda_1^n + a_2\lambda_2^n + a_3\lambda_3^n$. The coefficients $a_{0\to3}$ are linear functions of S^0, defined by the matrix U. By the least-squares approximation, the experiment gives the values of $\lambda_{0\to3}$ and the coefficients $a_{0\to3}$. By solving the linear system of equations defining the $a_{0\to3}$ as a function of $S^0_{0\to3}$, we always find a solution $S^0_{0\to3}$ in good agreement with the experimental results defined by $\lambda_{0\to3}$ and $a_{0\to3}$. For this reason, all the recurrence laws defined by a matrix with the eigenvalues $\lambda_{0\to3}$ give exactly the same theoretical points by the least-squares experimental calculation. It is for this reason that we can make any permutation between the misses $\alpha_{0\to3}$. We can also make a permutation between lines and columns of the matrix, as we have made between the first model of Forbush *et al.* (1971) and our model.